DIGITAL IMAGE ANALYSIS OF REACTIVE OXYGEN SPECIES AND CA2+ IN MOUSE 3T3 FIBROBLASTS

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Abstract: Recently, analysis of digital images with confocal microscope has become a routine technique and indispensable tool for cell biological studies and molecular investigations. Because the light emitted from the point out-of-focus is blocked by the pinhole and can not reach the detector, thus only an image of the fluorescence from the focal plane is imaged. In present studies, we use the probes 2′, 7′-dichlorof luorescein diacetate (H2DCF-DA) and Fluo-3 AM to research reactive oxygen species (ROS) and Ca2+ in mouse 3T3 fibroblasts, respectively. Our results indicate that the distribution of ROS and Ca2+ were clearly seen in mouse 3T3 fibroblasts. Moreover, we acquired and quantified the fluorescence intensity of ROS and Ca2+ with Leica Confocal Software. It was found that the quantified fluorescence intensity of ROS and Ca2+ was 123.30.26±8.99 and 125.13±12.16, respectively. Taken together, our results indicate that it is a good method to research the distribution and fluorescence intensity of ROS and Ca2+ in cultured cells with confocal microscope.

Keywords: digital image, confocal microscope, mouse 3T3 fibroblasts, reactive oxygen species, Ca2+

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1. INTRODUCTION

Confocal microscope, which works by exciting fluorescence with a highly focused beam of laser light, is one of the most exciting advances in optical microscope. Recently, confocal microscope has become a routine technique and indispensable tool for cell biological studies and molecular investigations (Lichtman, 1994). Since there are two pinholes in confocal microscope, the light emitted from the point out-of-focus is blocked and can not reach the detector. Thus only a digital image of the fluorescence from the focal plane is observed. Moreover, the laser can scan from point to point over the sample and a single two-dimensional image of the optical section is acquired (Lichtman, 1994). Because sectioning is performed using optics rather than the physical sectioning of the sample, living cells can be analyzed with confocal microscope (Blancaflor and Gilroy, 2000).

Using confocal microscope, the cells can be analyzed in three dimensions with much more clarity than conventional microscope. There have been numerous scientific papers employing confocal microscope in cell biology, and this technology is very important for biology science research (Serhal et al., 2007; Wang et al., 2004; Lee et al., 2007). Confocal microscope has been used to investigate the heterogeneity of plant mitochondrial responses (Armstrong et al., 2006), Ca^{2+} level (Nichols et al., 2007), oxidative stress in cells (Kannan et al., 2006) and mitochondrial localization (Yang et al., 2006). Since confocal microscope is an appropriate and important method for quantitative and qualitative analysis of cells, we applied confocal microscope for analyzing the distribution of reactive oxygen species (ROS) and Ca^{2+} in mouse 3T3 fibroblasts in present studies.

2. MATERIALS AND METHODS

2.1 Cell culture

Mouse 3T3 fibroblasts, obtained from China Centre for Type Culture Collection (Wuhan, China), were maintained at 37°C with 5% CO2 and 95% air in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum.

2.2 Ca^{2+} detection

To analyze Ca2+ level, cells were loaded with Fluo-3 AM (Molecular Probes, 5 μmol/L) at 37°C for 30 minutes. Excess dye was eliminated by washing the disks three times in PBS buffer.

2.3 ROS detection

Intracellular production of ROS was visualized by using 2′, 7′-dichlorof luorescein diacetate (H_2DCF-DA, Molecular Probes). This nonpolar compound is converted to the membrane-impermeant polar derivative H_2DCF by esterases when it is taken up by the cell. H_2DCF is nonfluorescent. However, it is rapidly oxidized to the highly fluorescent DCF by intracellular ROS (Genty et al., 1989). For assessment of ROS, the samples of cells were immersed in 1.5 ml of 10 μM DCFH-DA for 50 min, wiped to remove excess solution, and placed inside the growth chamber. At the end of the incubation period, the cells were rinsed briefly with PBS buffer.

2.4 Digital Images acquirement with confocal microscope

For microscopy, Cells plated on glass-bottom dishes were loaded with fluorescent probes as described above. After washing the dyes, the cells were put under an inverted microscope (Leica DM IRE 2, Germany). A laser-scanning confocal microscope (Leica TCS SP2, Germany) with an air-cooled, argon-ion laser as the excitation source at 488 nm was used to view the sites of ROS and Ca^{2+}, respectively.

Images were obtained with a 100× oil immersion objective lens. ROS and Ca2+ were respectively detected in the green and red channel. The channel settings of pinhole, detector gain, amplification offset and gain were adjusted to provide an optimal balance of fluorescent intensity of the targeted cells and background. Data were collected by a computer attached to the instrument, stored on the hard drive, processed with a Leica TCS Image Browser, and transferred to Adobe Photoshop 6.0 for preparation of figures.

2.5 Digital Images analysis with confocal software

The fluorescence intensity of ROS and Ca2+ in mouse 3T3 fibroblasts was acquired and quantified with Leica Confocal Software. Data are expressed as mean ± SD.

3. RESULTS AND DISCUSSION

Reactive oxygen species (ROS), which include superoxide ($\cdot O_2^-$), the hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), are produced by metabolic activation of molecular oxygen (Kohen and Nyska, 2002). Under normal metabolic activities such as respiration and photosynthesis, ROS are

also produced. However, the production of ROS is enhanced during stresses such as nutrient limitation, exposure to xenobiotics and rehydration. Cells have evolved a variety of mechanisms to counteract the effects of reactive oxygen species, which include antioxidant enzymes and low-molecular-weight antioxidants (Scandalios, 1993; Kohen and Nyska, 2002). If not effectively and rapidly removed from cells, ROS will damage a wide range of macromolecules, possibly leading to cell death. Because ROS play an important part in a variety of biotic and abiotic stress conditions, the methods for direct in vivo identification and quantification of ROS is of special importance in stress studies.

H_2DCF-DA, which is not oxidized by superoxide, can be oxidated to fluorescent DCF by H_2O_2 and organic peroxides (Zhu et al., 1994). It has been found that H_2DCF-DA is a specific probe for intracellular H_2O_2 in a wide variety of organisms to study the mitochondrial H_2O_2 production (Pantopoulos et al., 1997; Quillet-Mary et al., 1997). Mitochondria are a major source of ROS in eukaryotic cells. In humans, aberrant mitochondrial ROS formation has been associated with conditions such as Parkinson's disease, amyotrophic lateral sclerosis, and aging (Beal, 1995). Our results indicate that ROS were produced intracellularly in mouse 3T3 fibroblasts, and was visualized clearly by laser-scanning confocal microscopy with the probe DCFH-DA (Fig.1. a).

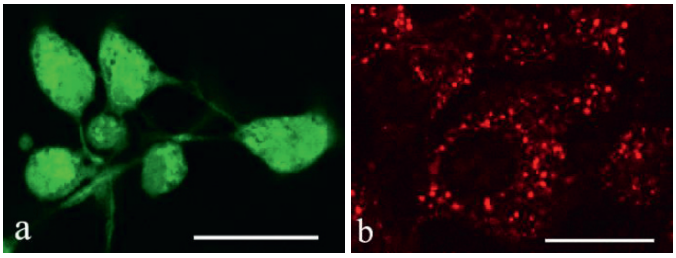

Fig.1. Intracellular localization of ROS and Ca^{2+} level. Laser-scanning confocal microscope images of ROS and Ca^{2+} level in mouse 3T3 fibroblasts. Cells were labeled with H_2DCF-DA and Fluo-3 AM, respectively. (a) Green channel, showing ROS fluorescence; Scale bars, 30 μm. (b) Red channel, showing Ca^{2+} fluorescence. Scale bars, 21.4 μm.

Since calcium is an intracellular messenger, the techniques for measuring cytosolic free Ca^{2+} concentrations have been essential. Fluo-3 fluorescence depends on concentration of free Ca^{2+} (Kao et al., 1989; Minta et al., 1989). Using Fluo-3 AM as probes, the distribution of Ca^{2+} can be clearly seen in mouse 3T3 fibroblasts (Fig.1. b). Moreover, we acquired and quantified the fluorescence intensity of ROS and Ca^{2+} in mouse 3T3 fibroblasts with Leica Confocal Software. It was found that the quantified fluorescence intensity of ROS and Ca^{2+} was 1123.30.26±8.99 and 125.13±12.16, respectively (Fig. 2).

Taken together, our results indicate that it is a good method to research the cultured cells with confocal microscope. With the fluorescence probes

H_2DCF-DA and Fluo-3 AM, not only the distribution of ROS and Ca^{2+} can be acquired, but also the fluorescence intensity of ROS and Ca^{2+} can be analyzed.

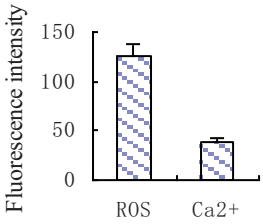

Fig.2. The fluorescence intensity of reactive oxygen species (ROS) and Ca^{2+} in mouse 3T3 fibroblasts was acquired and quantified with Leica Confocal Software. The error bars represent the SD.

4. CONCLUSION

The developments in computer technology and reagents that brought fluorescence-based methods to microscopy have become prevalent in cell biology. Our results indicate that it is a good method to research the cultured cells with confocal microscope. With the fluorescence probes H_2DCF-DA and Fluo-3 AM, not only the distribution of ROS and Ca^{2+} can be acquired, but also the fluorescence intensity of ROS and Ca^{2+} can be analyzed.

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